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Purification and functional inactivation of the fission yeast MCM^{MCM-BP} complex

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ABSTRACT

The MCM (mini-chromosome maintenance) complex is the core of the eukaryotic replicative helicase and comprises six proteins, Mcm2–Mcm7. In humans, a variant form of the complex has Mcm2 replaced by the MCM–BP protein. Recent results suggest that a similar complex exists in fission yeast with an essential role in DNA replication and cell cycle progression. Here, we describe the purification and subunit composition of the fission yeast MCM^{Mcb1} complex. Using newly generated temperature-sensitive alleles, we show that loss of MCM^{Mcb1} function leads to accumulation of DNA damage, checkpoint activation and cell cycle arrest, and provide evidence for a role for MCM^{Mcb1} in meiosis.

Structured summary of protein interactions:
Mcb1 physically interacts with Mcm4 by pull down (View interaction)
Mcb1 physically interacts with Mcm5 by pull down (View interaction)
Mcb1 physically interacts with Mcm6 by pull down (View interaction)
Mcm4 and **Mcb1** physically interact by bimolecular fluorescence complementation (View interaction)
Mcb1 physically interacts with Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 by tandem affinity purification (View interaction)
Mcb1 physically interacts with Mcm7 by pull down (View interaction)

1. Introduction

In all forms of cellular life, chromosomal DNA replication requires the complex interplay of a large number of essential and non-essential protein factors in a spatially- and temporally-coordinated manner. In eukaryotic cells, replication is initiated at multiple sites on each chromosome called replication origins [1]. These are bound by ORC (origin recognition complex), a conserved six-subunit protein complex. During G1, ORC binds to the Cdc6 protein and together these factors recruit Cdt1 and inactive MCM (mini-chromosome maintenance) DNA helicase to form the pre-replicative complex (pre-RC). Assembly of the pre-RC (known as replication licensing) is tightly regulated to ensure once-per-cell-cycle regulation of replication initiation. At the onset of S-phase, multiple additional factors are assembled to form the replisome, the molecular assembly that drives bidirectional movement of the replication forks [1].

Many lines of evidence point to the MCM complex being the catalytic core of the eukaryotic replicative helicase. Cdc45 and GINS interact with MCM to form a ternary complex (known as the CMG (Cdc45–MCM–GINS) complex) with robust DNA helicase activity [2–4]. MCM itself comprises six related protein subunits, Mcm2–Mcm7 [5,6]. Each is a member of the AAA+ family [7,8] of ATPase and ATP-binding proteins and is essential for chromosomal replication. The heterohexameric MCM complex forms a ring structure [5] capable of encircling single- or double-stranded DNA but the mechanism by which MCM unwinds DNA in cells is unclear [9,10].

While the primary focus of MCM-related research has been on the function of the canonical MCM complex, several related protein complexes have also been identified [11]. In mammalian cells [12], in *Xenopus* [13], in plants [14,15] and most recently, in the fission yeast *Schizosaccharomyces pombe* [16], an alternative MCM complex (MCM^{MCM-BP}) is formed when Mcm2 is replaced by MCM–BP. The function of this complex is unclear, although it has been suggested that it might function to facilitate unloading of the canonical MCM complex from chromatin in late S-phase [13].

Abbreviations: MCM, mini-chromosome maintenance; ORC, origin recognition complex; pre-RC, pre-replicative complex; CMG, Cdc45–MCM–GINS; TAP, tandem affinity purification

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In the fission yeast, the essential *mcb1*$^{+}$ gene encodes an MCM–BP homologue. Deletion of *mcb1*$^{+}$ results in cell cycle arrest and concomitant cell elongation, with the arrested cells having a 2C DNA content and for the most part, a single nucleus [16]. Mcb1 is an abundant protein, present throughout the cell cycle and enriched in the nucleus, that can be co-immunoprecipitated with Mcm3, Mcm4, Mcm6 and Mcm7 but not with Mcm2. Overexpression of Mcb1 causes S-phase inhibition, accumulation of Rad22 repair foci and activation of the Chk1-dependent DNA damage checkpoint, most likely by disrupting normal MCM function [16].

The co-immunoprecipitation data described above is consistent with the presence in fission yeast of an MCM^{MCM-BP} complex similar to that seen in human cells, comprising Mcb1, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7. Here we present tandem affinity purification of this complex and identification (by mass spectrometry) of Mcb1, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 as its only subunits, thus confirming the predicted subunit composition. In addition, we have isolated temperature-sensitive alleles of *mcb1* and use these to demonstrate that inactivation of Mcb1 function leads to accumulation of Rad22 DNA repair foci and activation of the Chk1-dependent DNA damage checkpoint, properties previously observed in Mcb1 overproducers. Mcb1 is also shown to play a role in meiosis in fission yeast, raising the possibility that the MCM^{MCM-BP} complex may function in meiosis in higher eukaryotes. These results highlight the vital role played by the MCM^{Mcb1} complex in fission yeast chromosome biology and reinforce the importance of fission yeast as a model system for studies aimed at shedding light on MCM^{MCM-BP} function.

2. Materials and methods

A detailed materials and methods can be found in the Supplementary data.

2.1. Fission yeast strains and methods

The fission yeast strains used are listed in Table 1. Standard fission yeast growth conditions and methods were used throughout. Cells were grown on YE or EMM medium. Matings were performed on low nitrogen EMM containing 1 g/l sodium glutamate.

2.2. Gene tagging and gene deletion

Gene tagging was accomplished by the PCR-mediated gene targeting method [17] in wild-type haploid strains *h*$^{-}$, *h*$^{+S}$ and *leu1-32 ura4-D18 h*$^{-}$ (Table 1).

2.3. Tandem affinity purification

Tandem affinity purification (TAP) was performed from cultures of exponentially-growing wild-type or *mcb1-TAP* cells essentially as described previously [18]. Samples for mass spectrometry analysis were excised from SDS–PAGE gels and processed by the University of St. Andrews Mass Spectrometry and Proteomics Facility as described in the Supplementary data.

2.4. Scale-scale co-purification assays

Native proteins extracts from mid-exponential phase cultures were prepared using a cell disruptor, cleared by centrifugation and added to activated IgG-sepharose beads. Following washing, bound proteins were subjected to SDS–PAGE and immunoblotting using anti-myc primary and HRP-linked sheep anti-mouse secondary antibodies. See Supplementary data.

2.5. Sucrose gradient centrifugation

Native proteins extracts of *mcb1-TAP*, *mcm4-TAP* and *mcm6-TAP* strains (see Table 1) were prepared as above, applied to 5–18% sucrose gradients and centrifuged for 22 h at 60000*g*. Gradient fractions were then collected, subjected to SDS–PAGE and immunoblotted with anti-TAP antibodies.

2.6. Microscopy

Live cells were stained with Hoechst 33342 and visualised on a Zeiss Axiovert 40CFL fluorescence microscope. DAPI (4′,6-diamidino-2-phenylindole) was used to stain DNA in fixed spores. Localisation of Mcb1-YFP in live cells was visualised using a DeltaVision microscope (see Supplementary data, Fig. S2).

2.7. PCR mutagenesis

Temperature-sensitive mutants were generated and identified at 35 °C as described [19] with *mcb1-YFP-natMX6* genomic DNA as the starting template.

2.8. Sensitivity to DNA damaging agents

Wild-type, *mcb1-Ts1* and *mcb1-Ts6* strains were spotted onto YE medium containing various DNA damaging agents, as indicated in the Supplementary data.

3. Results

3.1. Purification and subunit composition of the fission yeast MCM^{Mcb1} complex

Tandem affinity purification [20] was used to purify the MCM^{Mcb1} complex from cells expressing a C-terminally TAP-tagged Mcb1 protein from its normal chromosomal locus (see Section 2). The purified material was resolved on SDS–PAGE, stained with silver and the identity of purified species identified by tandem mass spectrometry (Fig. 1A). This approach identified the Mcb1–TAP, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 proteins (see Supplementary data, Table S1, for MASCOT scores). Mcm2 was not detected. In parallel experiments, the MCM^{Mcm2} complex was purified from cells expressing a C-terminally TAP-tagged Mcm2 protein; no Mcb1 protein was detected in these preparations (Supplementary data, Fig. S1A). To investigate the size of the Mcb1 complex protein extracts were fractionated by sucrose gradient sedimentation. The Mcb1 protein displayed a broad sedimentation peak from ~400 to 200 kDa, similar to the patterns displayed by Mcm4 and Mcm6 (Fig. 1B).

To confirm these interactions, Mcb1–TAP was purified from small-scale cultures of cells carrying C-terminal myc epitope-tagged Mcm2, Mcm4, Mcm5, Mcm6 and Mcm7 proteins expressed from their normal chromosomal loci and the ability of the myc-tagged proteins to co-precipitate with Mcb1–TAP analysed by immunoblotting following pull-down using IgG-Sepharose. Consistent with the results from the large-scale TAP purification, Mcb1 was shown to co-precipitate with Mcm4-13myc, Mcm5-13myc, Mcm6-13myc and Mcm7-13myc but not Mcm2-13myc (Fig. 1B). In additional experiments, Mcb1-13myc failed to co-purify with Mcm2-TAP while Mcm4-13myc did (Supplementary data, Fig. S1B).

Bimolecular fluorescence complementation (BiFC) has been used to demonstrate interactions between MCM and GINS subunits in fission yeast [21]. This approach is based on the ability of N- and C-terminal fragments (VN173, VC155 respectively) of a modified

Table 1
Fission yeast strains.

Strain number	Genotype	Reference or source
Sp347	*h*$^-$	[a]
Sp348	*h*$^{+S}$	[b]
Sp535	*mcb1-TAP-natMX6 h*$^-$	This study
Sp550	*mcm2-13myc-kanMX6 h*$^-$	This study
Sp545	*mcm4-13myc-kanMX6 h*$^-$	This study
Sp648	*mcm5-13myc-kanMX6 h*$^-$	This study
Sp599	*mcm6-13myc-kanMX6 h*$^-$	This study
Sp546	*mcm7-13myc-kanMX6 h*$^-$	This study
Sp547	*mcb1-TAP-natMX6 mcm2-13myc-kanMX6 h*$^-$	This study
Sp548	*mcb1-TAP-natMX6 mcm4-13myc-kanMX6 h*$^-$	This study
Sp623	*mcb1-TAP-natMX6 mcm5-13myc-kanMX6 h*$^-$	This study
Sp600	*mcb1-TAP-natMX6 mcm6-13myc-kanMX6 h*$^-$	This study
Sp549	*mcb1-TAP-natMX6 mcm7-13myc-kanMX6 h*$^-$	This study
Sp585	*mcm2-TAP-natMX6 h*$^-$	This study
Sp586	*mcb1-13myc-natMX6 h*$^{+S}$	This study
Sp594	*mcm2-TAP-natMX6 mcb1-13myc-natMX6*	This study
Sp530	*mcb1-VN173-kanMX6 h*$^{+S}$	This study
Sp489	*mcm4-VC155-natMX6 h*$^-$	[c]
Sp561	*mcb1-VN173-kanMX6 mcm4-VC155-natMX6*	This study
Sp322	*leu1-32 ura4-D18 h-*	This study
Sp532	*mcb1-YFP-natMX6 h*$^-$	This study
Sp578	*mcb1-Ts1-YFP-natMX6 leu1-32 ura4-D18 h-*	This study
Sp580	*mcb1-Ts6-YFP-natMX6 leu1-32 ura4-D18 h-*	This study
Sp651	*mcb1-Ts5-YFP-natMX6 leu1-32 ura4-D18 h-*	This study
Sp617	*mcb1-Ts1-YFP-natMX6 rad22-YFP-kanMX6 h+*	This study
Sp618	*mcb1-Ts6-YFP-natMX6 rad22-YFP-kanMX6 h+*	This study
Sp582	*rad22-YFP-kanMX6 leu1-32 ura4-D18 h-*	[d]
Sp626	*mcb1-Ts1-YFP-natMX6 chk1::ura4+ leu1-32 ura4-D18*	This study
Sp625	*mcb1-Ts1-YFP-natMX6 cds1::ura4+ leu1-32 ura4-D18*	This study
Sp628	*mcb1-Ts6-YFP-natMX6 chk1::ura4+ leu1-32 ura4-D18*	This study
Sp627	*mcb1-Ts6-YFP-natMX6 cds1::ura4+ leu1-32 ura4-D18*	This study
Sp336	*cds1::ura4 ura4-D18 leu1-32 h+*	[e]
Sp338	*chk1::ura4 ura4-D18 leu1-32 h+*	[f]

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yellow fluorescent protein (YFP) to form a fluorescent complex when brought together by the association of two interacting partners. To test whether this approach could be used to analyse interactions between Mcb1 and Mcm4, the Mcb1 protein was tagged at its C-terminal end with the VN173 protein. Cells co-expressing Mcm4-VC155 and Mcb1-VN173 displayed bright nuclear fluorescence (Fig. 1C) that was not seen in cells expressing either fusion protein alone. Consistent with this, we found that Mcb1 C-terminally tagged with full-length YFP was localised at least in part to the nucleus (Supplementary data, Fig. S2), consistent with the large-scale protein localisation screening results [22].

3.2. Inactivation of MCM^{Mcb1} leads to accumulation of Rad22 repair foci

To analyse MCM^{Mcb1} function in greater detail, a number of temperature-sensitive *mcb1* alleles were generated by random mutagenesis (see Section 2). Three alleles were chosen for further analysis: *mcb1-Ts1, mcb1-Ts5* and *mcb1-Ts6*. All three carry mutations in the C-terminal domain of Mcb1 (Fig. 2A). Cells carrying either *mcb1-Ts1*, *mcb1-Ts5* or *mcb1-Ts6* are incapable of colony formation at 35 °C (Fig. 2B). The arrested cells are elongated (Fig. 2C) and have a 2C DNA content (Supplementary data, Fig. S3), as previously reported for *mcb1Δ* cells [16]. Neither *mcb1-Ts1* nor *mcb1-Ts6* displayed increased sensitivity to UV light or to the DNA damaging agents camptothecin, methyl methanesulfonate (MMS) or 4-nitroquinoline 1-oxide (4NQO) when grown at permissive or semi-permissive temperatures (Supplementary data, Fig. S4).

The consequences of loss of Mcb1 function were examined using strains expressing Rad22-YFP. Rad22 is the fission yeast orthologue of budding yeast Rad52 which binds ssDNA during homologous recombination and at double-strand breaks (DSBs) or other sites where ssDNA is present, leading to the formation of foci of Rad52-YFP (Rad22-YFP) fluorescence visible under the microscope [23]. To analyse the effects of Mcb1 inactivation on focus formation, *mcb1-Ts1 rad22-YFP* and *mcb1-Ts6 rad22-YFP* strains were constructed and Rad22-YFP foci in cells grown at the permissive and restrictive temperatures counted (Fig. 2D and E). Even at the permissive temperature, the number of Rad22 foci visible in *mcb1* cells was increased compared to wild-type. After 6 h at the restrictive temperature over 60% of cells displayed foci compared to ~15% of wild-type cells (Fig. 2D).

3.3. Inactivation of MCM^{Mcb1} leads to activation of the Chk1-dependent DNA replication checkpoint

The elongated cell phenotype seen with temperature-sensitive *mcb1* strains is likely due to activation of either the DNA replication or DNA damage checkpoint pathways that centre, respectively, on the Cds1 and Chk1 checkpoint kinases. To examine this, *mcb1-Ts1* and *mcb1-Ts6* strains were crossed to *chk1Δ* and *cds1Δ* mutants and the behaviour of double mutants analysed following temperature shift to 35 °C for 6 h.

Both *mcb1 cds1Δ* double mutant strains elongated at 35 °C in a manner that was indistinguishable from *mcb1* single mutants. Cell viability (measured by ability to form colonies when plated at the permissive temperature of 25 °C) was largely unaffected by the temperature shift (Fig. 3A). The Cds1 kinase is therefore not required for cell cycle arrest in the absence of MCM^{Mcb1}. In sharp contrast, the *mcb1 chk1Δ* mutant strains failed to elongate at 35 °C and rapidly lost viability (Fig. 3A). DAPI staining of cell nuclei identified examples of chromosome segregation and cell division defects consistent with entry into mitosis with incompletely replicated chromosomes (Fig. 3B). Taken together, the results indicate that the cell cycle arrest observed in the absence of fully functional MCM^{Mcb1} is dependent on activation of the Chk1-dependent DNA damage checkpoint.

3.4. MCM^{Mcb1} function in meiosis

MCM activity is essential for pre-meiotic S-phase in fission yeast. To test whether this was also likely to be true of MCM^{Mcb1}, *mcb1-Ts1* and *mcb1-Ts5* strains of opposite mating type were self-crossed at permissive (25 °C) and semi-permissive (30 °C) temperatures and the products of meiosis examined microscopically following DAPI staining (Fig. 4). At the semi-permissive temperature of 30 °C, 25% and 29% of spores from *mcb1-Ts1* and *mcb1-Ts5* crosses, respectively, displayed abnormal phenotypes, in contrast to ~2% in self-crosses of wild-type strains. At the permissive temperature of 25 °C, <2% of all spores displayed abnormalities for all crosses.

4. Discussion

Understanding the biology of chromosomal DNA replication requires a detailed understanding of the molecular components of the replication machinery. The MCM helicase functions at the

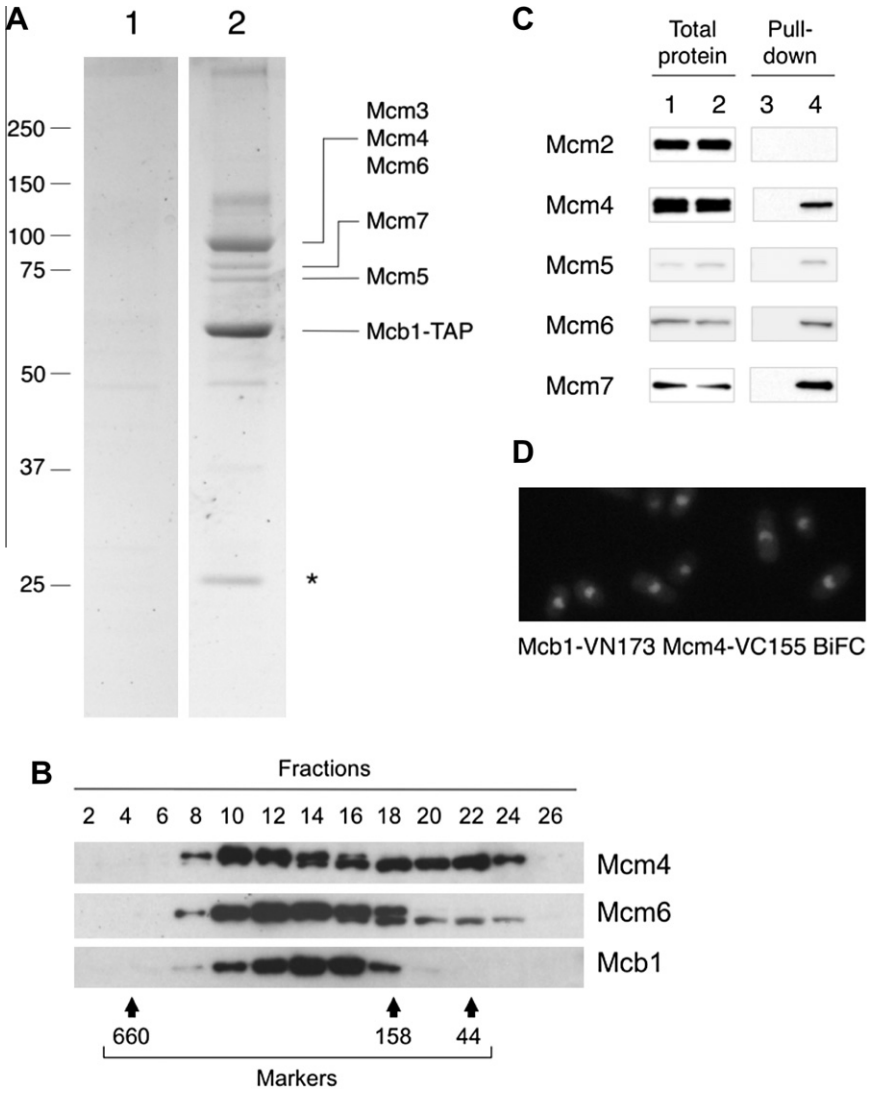

Fig. 1. MCM^{Mcb1} complex and interactions. (A) Tandem affinity purification (TAP) from wild-type (lane 1) and *mcb1-TAP* (lane 2) cells. Purified samples were subjected to SDS–PAGE and silver staining. Purified species were identified by mass spectrometry. (B) Sucrose gradient centrifugation (5–18%) of extracts prepared from *mcm4-TAP*, *mcm6-TAP* and *mcb1-TAP* strains. Gradient fractions were subjected to SDS–PAGE and immunoblotting with anti-TAP antibodies. Sedimentation positions of the marker proteins thyroglobulin (660 kDa), aldolase (158 kDa) and ovalbumin (44 kDa) are shown. (C) Co-purification of Mcb1–TAP with Mcm4-13myc, Mcm5-13myc, Mcm6-13myc and Mcm7-13myc, but not Mcm2-13myc. Extracts prepared from double tagged strains (Table 1) were incubated with IgG-sepharose and the bound material subjected to SDS–PAGE and immunoblotting with anti-myc antibodies. Left: soluble protein extracts. Right: pulled-down material. (D) Interaction between Mcb1 and Mcm4 visualised by bimolecular fluorescence complementation (BiFC) in an *mcb1-VN173 mcm4-VC155* strain.

heart of this machinery as the catalytic core of the CMG complex, the enzyme complex that unwinds duplex DNA ahead of the replication fork [3]. MCM is composed of six related subunits, Mcm2–Mcm7, each of which is a member of the AAA+ superfamily of ATPases [7,8]. To date, three additional proteins related to MCM have been identified in eukaryotes: MCM8, MCM9 and MCM–BP. *Drosophila* MCM8 plays an essential role in promoting crossover formation during meiosis [24,25] while *Xenopus* MCM9 has been shown to interact with Cdt1 and be required for pre-RC formation [26–28]. Despite being widely conserved across eukaryotic evolution, neither MCM8 nor MCM9 is present in either of the budding yeast *Saccharomyces cerevisiae* or the distantly-related fission yeast *S. pombe* [29]. In contrast, MCM–BP is present in *S. pombe* but not *S. cerevisiae*. The function of MCM–BP remains unclear but evidence from humans [12] and *Xenopus* [13] indicates that this protein replaces Mcm2 to form an alternative MCM complex and recent results from fission yeast show the protein to be essential for cell cycle progression [16].

Here, we provide confirmation that the fission yeast MCM^{MCM-BP} complex is composed of the MCM–BP homologue protein Mcb1 together with Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 (Fig. 1) and that Mcm2 is not a part of the MCM^{Mcb1} complex. Thus the situation in fission yeast appears to resemble that in human cells [12] and in *Xenopus* [13] rather than as has been observed in plants, where MCM–BP appears to co-purify with all six MCM subunits [14]. The function of the MCM^{Mcb1} complex in fission yeast remains uncertain but it is clear that both inactivation (this paper) and overproduction [16] of Mcb1 is highly detrimental to cells, causing the accumulation of DNA repair foci (Fig. 2), activation of the Chk1-dependent DNA damage checkpoint (Fig. 3) and cell cycle arrest. At least part of the explanation for the effects of overproduction seems to lie with disruption of normal MCM complex function [16]. As it seems unlikely that disrupting Mcb1 function using temperature-sensitive *mcb1* alleles will have an indirect effect on the normal MCM complex, we are currently focusing our efforts on characterising in detail the properties of

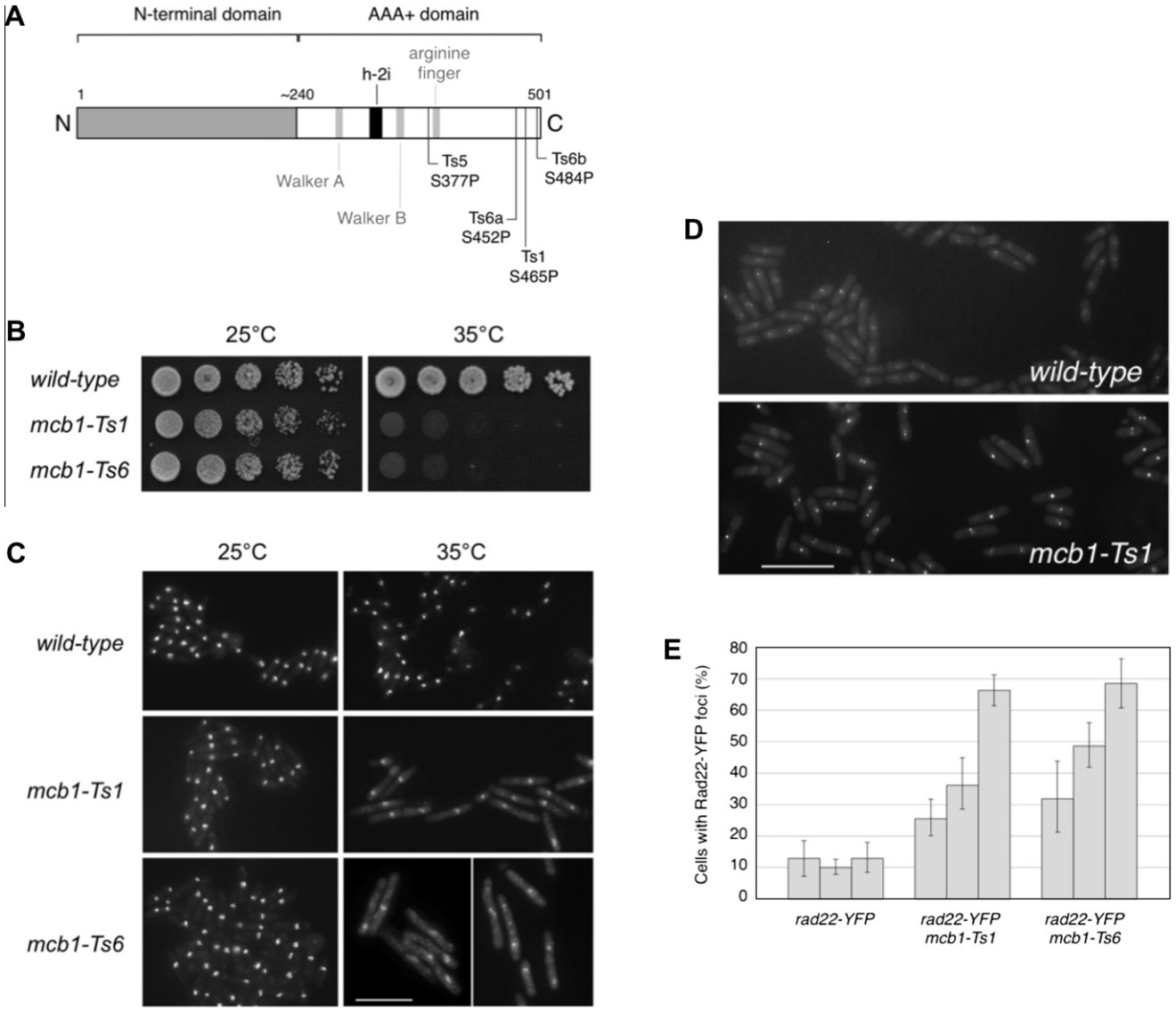

Fig. 2. Temperature-sensitive *mcb1* mutants. (A) Schematic of fission yeast Mcb1 protein showing location of point mutations in the temperature-sensitive *mcb1-Ts1*, *mcb1-Ts5* and *mcb1-Ts6* alleles. The approximate locations of the Walker A, Walker B, arginine finger and helix 2 insert (h2-i) features characteristic of the canonical MCM proteins (but absent in MCM–BP) are shown for reference. (B) Serial dilution spots of wild-type, *mcb1-Ts1* and *mcb1-Ts6* strains at 25 and 35 °C. (C) Hoechst 33342 DNA staining in *mcb1-Ts1* and *mcb1-Ts6* strains at 25 °C and after 6 h at 35 °C. Scale bar: 20 μM. (D) Rad22-YFP DNA repair foci visualised in wild-type and *mcb1-Ts1* cells following incubation at 35 °C for 6 h. Scale bar: 20 μM. (E) Quantitation of Rad22-YFP foci in wild-type, *mcb1-Ts1* and *mcb1-Ts6* at 25 °C and after 4 and 6 h at 35 °C (from left to right in each set of three). Standard deviations are shown.

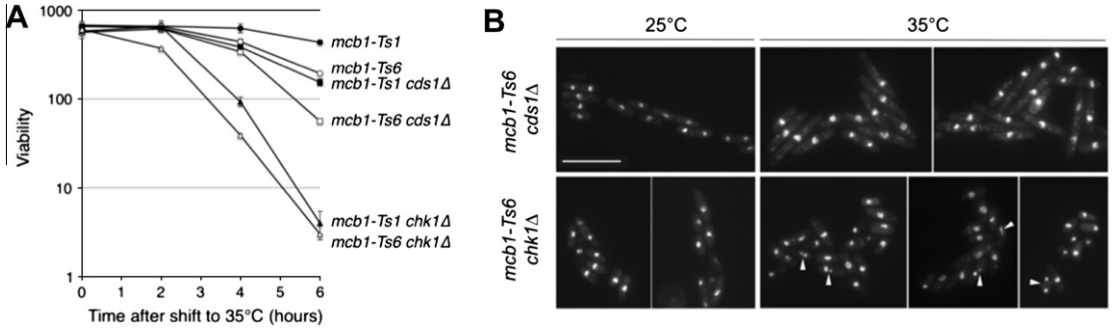

Fig. 3. Cell cycle arrest in *mcb1* mutants is DNA damage checkpoint-dependent. (A) Survival curves for *mcb1-Ts*, *mcb1-Ts chk1Δ* and *mcb1-Ts cds1Δ* strains following shift to 35 °C for 6 h. (B) Hoechst 33342 DNA staining of *mcb1-Ts6 cds1Δ* and *mcb1-Ts6 chk1Δ* at 25 °C and following shift to 35 °C for 6 h. *mcb1-Ts6 chk1Δ* cells displaying nuclear abnormalities are indicated by the white arrowheads. Scale bar: 20 μM. See text for details.

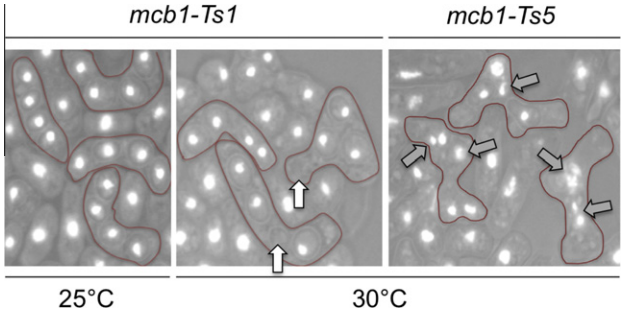

Fig. 4. MCM^{Mcb1} function is required in meiosis. DAPI-stained asci produced from *mcb1-Ts1* and *mcb1-Ts5* self-crosses at 25 or 30 °C are shown, with the walls of selected asci outlined in dark red. White arrows indicate anucleate spores (no DAPI staining); grey arrows indicate abnormal nucleoid structures (stained with DAPI).

the temperature-sensitive *mcb1* strains in order to delineate the function of the MCM^{Mcb1} complex.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.10.033.

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